THE EMBO JOURNAL

Manuscript EMBO-2015-93489

DnaJ/Hsc70 chaperone complexes control the extracellular release of neurodegenerative-associated proteins

Sarah N Fontaine, Dali Zheng, Jonathan J Sabbagh, Mackenzie D Martin, Dale Chaput, April Darling, Justin H Trotter, Andrew R Stothert, Bryce A Nordhues, April Lussier, Jeremy Baker, Lindsey Shelton, Mahnoor Kahn,Laura J Blair, Stanley M Stevens, Jr, Chad A Dickey

Corresponding author: Chad Dickey, University of South Florida

Review timeline:

Submission date:	16 November 2015
Preconsultation	16 December 2015
Editorial Decision:	17 December 2015
Revision received:	14 March 2016
Editorial Decision:	04 April 2016
Revision received:	25 April 2016
Accepted:	28 April 2016

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Preconsultation 16 December 2015

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see below, the referees find the findings interesting. However, they also find that the analysis needs to be significantly extended for consideration here. As it is unclear if you can address the key concerns, I have therefore decided to do sort of a pre-consultation to see what you can do within a reasonable timeframe (3-6 months) before taking the decision on the manuscript. I think this is the most productive way forward at this stage.

The referees raise a number of technical points - like you need to use a more quantitative method instead of the dot blot analysis (if ELISA is not possible/feasible then western blot) and to repeat some of the experiments in neuronal cells etc. I presume that these experiments should be doable. Referee #1 brings up one important concern in that while the data support that DNAJC5 interacts with Tau and HSC70 to mediate release this is not proven. At the moment it is still possible that DNAJC5 and HSC70 acts via independent pathways. This would have to be addressed by blocking DNAJC5 interaction with Hsp70 to see if this affects release. Co-localization is not enough. I don't know if you have tried such experiments and if they are doable, but we would need such levels of insight to support the conclusions drawn. I don't expect you to address all the concerns raised by referee #3, but the experiment mentioned above is important.

Would be good if you could send me a constructive point-by-point response that would outline what experiments you can undertake to address the concerns raised. We can also discuss further by phone if that is helpful.

REFEREE REPORTS

Referee #1

The manuscript by Fontaine and colleagues explores the relationship between DNAJC5 and extracellular protein aggregate release. The question is intriguing and relevant and as yet there is no clear understanding as to the mechanism of protein release. This is particularly relevant to many neurodegenerative disorders with spreading pathology. Unfortunately the connection between DNAJC5, Hsp70 and tau is not fully sorted out and lacks proof that the three act together.

Concerns:
Figure 1: Why is a regular western not utilized? Is the dot blot meant to convey that the extracellular tau is "aggregated"

Figure 2B : Why is there secreted synuclein in the empty vector control. In addition, It would be nice to see the where these proteins localize. All three of these proteins, tau, synuclein and TDP-43 likely localize to different cellular compartemnts. So how is nuclear TDP-43 getting secreted. What is the intracellular state of these proteins "soluble" aggregated.

Figure 3: It looks like there is more intracellular and extracellular tau. Has the level of tau experssion been confirmed as similar with and without dnajc5 in these cultures?

Figure 4: it is unclear to me what this figure is telling me? it seems to me that if there was more tau at the synapses or less tau at the synapse one could make a similar conclusion.

Figure 5-7: what is the inhibitor that is used? This only shows that HSc70 is required for tau release and that DNAJC5 is required. tau needs to bind to Hsc70 and DNAJC5 needs to get to the membrane for tau release but there is no experiment or evidence that DNAJC5 interacts with Tau and HSC70 to mediate release. These data could suggest that these are two independent pathways.

Referee #2

In this manuscript Fontaine et al propose that DnaJC5, a co-chaperone, works with the molecular chaperone Hsc70 to regulate the extracellular release of tau and other neurodegenerative proteins such as TDP43 and alpha-synuclein. They report that this DnaJC5/Hsc70 mediated release is a unifying mechanism by which neurodegenerative proteins exit the cell to propagate pathology throughout the brain.

Overexpression of DnaJC5 in HEK cells leads to an increase in extracellular tau, alpha-synuclein and TDP43. Overexpression of DnaJC5 in neuronal cell lines and organotypic slice cultures leads to an increase in extracellular tau. The authors report that this DnaJC5 mediated release of tau is dependent on both tau's interaction with Hsc70 and on the activity of Hsc70 itself. Blocking both abolishes increase in extracellular tau. Finally they report that other DnaJ proteins do not affect release of tau as which occurs with DnaJC5.

Overall, the findings with HSC70 and DnaJC5 modulate the extracellular release of tau, synuclein, and TDP-43 is novel and suggest that they could be involved in the spreading of these proteins. However, only the normal, non-aggregated forms of these proteins are assessed here. These findings add importantly to this area. Several additional points need to be addressed for the data to be better interpretable.

Comments:

1. While the dot blots and western blots do show increased amount of extracellular proteins, it would be better to have a more quantitative method to better quantitate at least the results for tau. This is particularly true for analyzing intracellular tau and for making meaningful comparisons between extra and intra-cellular levels of these proteins. For example, in Fig 6B, there seems to be very little or no difference in extracellular tau (as seen in the dot blot) in the absence and presence of DnaJC5, which seems to go against the main premise of the paper. Quantifying these levels would be more accurate. In addition, showing a representative Western blot of tau, alpha-synuclein, and TDP-43 for the proteins in the extracellular space (instead of a dot blot), would be useful.

2. Experiments showing increases in extracellular alpha-synuclein and TDP43 were done only in HEK cells. In order for the authors to make the claim that DnaJC5/Hsc70 drive release of multiple proteins, it would be helpful to show the same experiments they did with tau for alpha-synuclein and TDP 43 in both HEK cells and a neuronal cell type.

3. Comparing effects of DnaJC5 on extra and intracellular levels of tau in Fig 1 and 3B, it seems that intracellular tau levels are reduced in HEK cells but not in M17 cells. Fig 3C also seems to indicate that DnaJC5 doesn't affect intracellular tau levels in primary neurons. A similar result is seen in the organotypic slice culture experiments shown in Fig 5. Why is this? The authors should comment on this.

4. Tau mRNA levels were only assessed in HEK cells to show that DnaJC5 mediated release of tau does not alter mRNA. Is this true for other cell types used as well? Similar analysis should be done on M17 cells as well as the other cell models used here.

Minor comments:

5. Methods section only includes methods for preparing tau plasmids. Authors should include information for preparation of other plasmids (a-synuclein, TDP43) as well.

6. Since many different cell lines/models were used in different figures, figure legends should clearly indicate which cell type was used for which experiment. It is often unclear (e.g. Fig 2, 6) which cell line was used.

7. Significance markers (asterisks) are missing from several panels. Is this because statistics were not done on that data or is it because the difference isn't significant? For example, see Fig 1B, 3C, 7B.

8. Minor grammatical error in page 8. "It possible" should read "It is possible..."

Referee #3

Reviewer Comments for EMBOJ-2015-93489

General summary and Opinion:
In their presented study "DNAJ/HSc70 chaperone complexes control extracellular release of neurodegenerative associated proteins", Sarah N. Fontaine et al. report that DNAJc5 and Hsc70 synergistically mediate the release of tau in vitro. The finding that chaperones are directly involved in shutteling tau out of the cell is in intriguing finding and very interesting idea; especially in the face of recently reported release and propagation of endogenous and wild-type transgenic tau. After showing that DnaJC5 overexpression triggers the release of also overexpressed tau in HEK cells, the authors show that this is true as well for misfolding mutant tau R406W and P301L, as well as for wild-type and mutant synuclein and TDP43. They continue to show a similar effect in neuron-like M17 cells and slice cultures from wild-type mice after AAV DnaJC5 transduction and a decrease in tau release in slices from DnaJC5 knockout mice.
In primary neuronal cultures (not clear from what source) tau and Hsc70 co-localization with presynaptic markers is indicated by immunofluorescence, and further show in au overexpressing HEK cells and neurons that tau release by DnaJC5 can be blocked when inhibiting Hsc70. Direct interaction of tau with DnaJC5 maybe needed as shown by mutant tau and DnaJC5 lacking interaction sites. And finally, intracellular degradation of tau by DnaJC7 counteracts tau release, as expected, by decreasing intracellular tau levels.

In the end, the authors speculate that DnaJ/Hsc70 mediated protein release is vesicle associated and could potential be a general mechanism for the release and propagation of misfolded or aggregated proteins in neurodegenerative diseases. They further speculate on synaptic co-release of tau, synuclein, and TDP43 in complex with DnaJ/Hsc70 in response to proteasome and/or degradation impairment.

The manuscript presented contains certainly some interesting ideas and presents a new perspective of chaperone activity in neurodegeneration, but seems in a too early state of the study; unfortunately the authors did not perform the right and carefully enough experiments to be convincing about their results and the observed phenomenon being actually relevant in neurons or even in vivo. The manuscript need major restoration and, at the stage the manuscript is in, I cannot support the publication in EMBO Journal.

Specific major concerns:
Most of the data is generated using HEK cells overexpressing tau and DnaJC5; such model is a good starting point for an initial observation of potential cellular mechanisms, however, if investigating a mechanism of CNS proteins involved in neuronal function, more relevant models - even in vitro - are necessary for proper translation of the relevance of the observed phenomenon. The detailed description the authors give in HEK cells has to be verified and translated into a neuronal systems, e.g. look at tau release in dependence on DnaJC5 and Hsc70 in primary neurons. Instead only minimum translation of the DnaJC5 facilitated tau release using neuron-like cells and slice cultures were performed. The representative data showed often is not convincing (e.g. no DnaJC5 expression in Figure 3A although presumably DnaJC5+ !?).

Furthermore, the techniques used to test the hypotheses and show the mechanisms are very limited and not always appropriate, here some examples:
To show the release of tau, synuclein and TDP43 is not just a result of general protein overexpression the proper controls are missing; for example, one or two non-IDP and non-aggregating proteins should be over-expressed and monitored for release in presence of DnaJCA.
Also it is claimed that DnaJC5 and Hsc70 form a complex with tau and are released as such; there is no experiment that supports this idea although it would be relatively straight forward to show the levels of DnaJC5/Hsc70 in the medium as well and further support this model by immunoprecipitation of one of the complex components. It could also easily be showed if tau and DnaJC5/Hsc70 are really released in vesicles performing a crude purification of membrane vesicles by centrifugation, and maybe doing immune-EM on the isolated vesicles; these are all standard techniques to proof the association with extracellular vesicles.

And what happens to endogenous tau from neurons or brain slices, is that released as well in vesicles in presence of DnaJC5? Overexpressing HEK cells have very different endo- and exocytosis characteristics compared to neurons, especially when they are starved in medium without serum they are known to release a lot of proteins when overexpressed.

Pre-synaptic localization of tau +/- DnaJC5 overexpression and co-localization of tau with Hsc70 is "proven" by immunohistochemistry (in only 2 sets of cultures); a simple synaptosome preparation of cultured neurons could actually show if that is true, whereas IHC is always prone for artifacts when used for quantitative intensity. The data is not convincing, especially since the synaptophysin staining is not restricted to the synapses (?) and the image insets are chosen too small to actually show what is stated.

The data is uniformly presented and analyzed as absolute released tau levels; I think it would be better to normalize these values to the actual intracellular tau expression levels since these may vary when overexpressing tau by transfection; showing the data as ratios extra:intra-cellular tau would give a clearer picture. Instead, tau is sometimes reduced or lacking intracellularly when there is (hence of course) also no tau release (e.g. Figure 3A, 5C, 6A, 7A), and it remains open and not discussed why that is the case.

Finally, if speculating about a mechanism of tau cell-to-cell transfer, experiments analyzing the transfer of tau and tau/DnaJC5/Hsc70-complexes to naïve cells should be performed.

Throughout the manuscript there is insufficient description of what has been done and what are the results; for example it is not clear which data is from HEK cells/MC17 or neurons, and it is often not very clear what kind of tau is used, what tau antibody is used for the detection, or what kind of neurons are cultured; inhibitors used for Hsc70 and the proteasome are not named and not described in methods. There are parts in methods that should be updated because data is not shown or shown data has no methods part.

The discussion is lacking critical comparing of the results with recent published data on tau release from neurons and glia as well as tau spreading.

All models showing the role of DnaJC5/Hsc70 for tau release have no legends, and it is not clear what is what.

Minor concerns:
Figure 1B is missing significance statement.
Figure 2 needs a non-aggregating protein control.

Figure 3A has no DnaJC5 in expressing cells; significance stars are too small.

Figure 3B needs an image showing actual AAV transduction of neurons in brain slices and not glia.

Figure 3C is missing significance statement.

Figure 3D has not indicated what the white tube is or the yellow ellipticals or "alpha"; legends to explain figure details are missing throughout all models presented!

Figure 5C is not clear what shows what here; needs to be reordered so that dot blot matches Western Blot; and why is there no intracellular tau in GFP+/Hsp70 inh+ neurons?

Figure 6A shows reduced intracellular tau with Hsp70 inhibitor in absence of DnaJC5. Why? - Please discuss!

Figure 6B shows very high tau release in absence of DnaJC5 but in presence of shRNA for Hsc70. Why? - Please discuss.

Figure 7A shows reduced intracellular tau with DnaJC8 an d DnaJB1, and hence no tau release. Why? - Please discuss!
Figure 7B is missing significance statement.

Supplemental Figure 2 - not clear why this experiment has been done and is worth showing; and siginificance statement is missing.

Supplemental Figure 3 - Dot blot of released tau in presence of different DnaJC5 concentrations is al saturated; unable to even see a dose dependence if there would be one! Please replace. Also give the name of the inhibitor used and it remains unclear why that experiment has been done.

1st Editorial Decision 17 December 2015

Thanks for sending me the point-by-point response and the phone discussion about what you can address in a revised version. Considering this input, I would like to invite you to submit a revised manuscript that addresses the key concerns raised. I should add that it is EMBO Journal policy to allow only one single round of major revision and that it is therefore important to address the key concerns at tis stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Fontaine and colleagues explores the relationship between DNAJC5 and extracellular protein aggregate release. The question is intriguing and relevant and as yet there is no clear understanding as to the mechanism of protein release. This is particularly relevant to many neurodegenerative disorders with spreading pathology. Unfortunately the connection between DNAJC5, Hsp70 and tau is not fully sorted out and lacks proof that the three act together.

Concerns:
Figure 1: Why is a regular western not utilized? Is the dot blot meant to convey that the extracellular tau is "aggregated"

Figure 2B : Why is there secreted synuclein in the empty vector control. In addition, It would be nice to see the where these proteins localize. All three of these proteins, tau, synuclein and TDP-43 likely localize to different cellular compartemnts. So how is nuclear TDP-43 getting secreted. What is the intracellular state of these proteins "soluble" aggregated.

Figure 3: It looks like there is more intracellular and extracellular tau. Has the level of tau experssion been confirmed as similar with and without dnajc5 in these cultures?

Figure 4: it is unclear to me what this figure is telling me? it seems to me that if there was more tau at the synapses or less tau at the synapse one could make a similar conclusion.

Figure 5-7: what is the inhibitor that is used? This only shows that HSc70 is required for tau release and that DNAJC5 is required. tau needs to bind to Hsc70 and DNAJC5 needs to get to the membrane for tau release but there is no experiment or evidence that DNAJC5 interacts with Tau and HSC70 to mediate release. These data could suggest that these are two independent pathways.

Referee #2:

In this manuscript Fontaine et al propose that DnaJC5, a co-chaperone, works with the molecular chaperone Hsc70 to regulate the extracellular release of tau and other neurodegenerative proteins such as TDP43 and alpha-synuclein. They report that this DnaJC5/Hsc70 mediated release is a unifying mechanism by which neurodegenerative proteins exit the cell to propagate pathology throughout the brain.

Overexpression of DnaJC5 in HEK cells leads to an increase in extracellular tau, alpha-synuclein and TDP43. Overexpression of DnaJC5 in neuronal cell lines and organotypic slice cultures leads to an increase in extracellular tau. The authors report that this DnaJC5 mediated release of tau is dependent on both tau's interaction with Hsc70 and on the activity of Hsc70 itself. Blocking both abolishes increase in extracellular tau. Finally they report that other DnaJ proteins do not affect release of tau as which occurs with DnaJC5.

Overall, the findings with HSC70 and DnaJC5 modulate the extracellular release of tau, synuclein, and TDP-43 is novel and suggest that they could be involved in the spreading of these proteins. However, only the normal, non-aggregated forms of these proteins are assessed here. These findings add importantly to this area. Several additional points need to be addressed for the data to be better interpretable.

Comments:

1. While the dot blots and western blots do show increased amount of extracellular proteins, it would be better to have a more quantitative method to better quantitate at least the results for tau. This is particularly true for analyzing intracellular tau and for making meaningful comparisons between extra and intra-cellular levels of these proteins. For example, in Fig 6B, there seems to be very little or no difference in extracellular tau (as seen in the dot blot) in the absence and presence of

DnaJC5, which seems to go against the main premise of the paper. Quantifying these levels would be more accurate. In addition, showing a representative Western blot of tau, alpha-synuclein, and TDP-43 for the proteins in the extracellular space (instead of a dot blot), would be useful.

2. Experiments showing increases in extracellular alpha-synuclein and TDP43 were done only in HEK cells. In order for the authors to make the claim that DnaJC5/Hsc70 drive release of multiple proteins, it would be helpful to show the same experiments they did with tau for alpha-synuclein and TDP 43 in both HEK cells and a neuronal cell type.

3. Comparing effects of DnaJC5 on extra and intracellular levels of tau in Fig 1 and 3B, it seems that intracellular tau levels are reduced in HEK cells but not in M17 cells. Fig 3C also seems to indicate that DnaJC5 doesn't affect intracellular tau levels in primary neurons. A similar result is seen in the organotypic slice culture experiments shown in Fig 5. Why is this? The authors should comment on this.

4. Tau mRNA levels were only assessed in HEK cells to show that DnaJC5 mediated release of tau does not alter mRNA. Is this true for other cell types used as well? Similar analysis should be done on M17 cells as well as the other cell models used here.

Minor comments:

5. Methods section only includes methods for preparing tau plasmids. Authors should include information for preparation of other plasmids (a-synuclein, TDP43) as well.

6. Since many different cell lines/models were used in different figures, figure legends should clearly indicate which cell type was used for which experiment. It is often unclear (e.g. Fig 2, 6) which cell line was used.

7. Significance markers (asterisks) are missing from several panels. Is this because statistics were not done on that data or is it because the difference isn't significant? For example, see Fig 1B, 3C, 7B.

8. Minor grammatical error in page 8. "It possible" should read "It is possible..."

Referee #3:

Reviewer Comments for EMBOJ-2015-93489

General summary and Opinion:
In their presented study "DNAJ/HSc70 chaperone complexes control extracellular release of neurodegenerative associated proteins", Sarah N. Fontaine et al. report that DNAJc5 and Hsc70 synergistically mediate the release of tau in vitro. The finding that chaperones are directly involved in shutteling tau out of the cell is in intriguing finding and very interesting idea; especially in the face of recently reported release and propagation of endogenous and wild-type transgenic tau. After showing that DnaJC5 overexpression triggers the release of also overexpressed tau in HEK cells, the authors show that this is true as well for misfolding mutant tau R406W and P301L, as well as for wild-type and mutant synuclein and TDP43. They continue to show a similar effect in neuron-like M17 cells and slice cultures from wild-type mice after AAV DnaJC5 transduction and a decrease in tau release in slices from DnaJC5 knockout mice.
In primary neuronal cultures (not clear from what source) tau and Hsc70 co-localization with presynaptic markers is indicated by immunofluorescence, and further show in au overexpressing HEK cells and neurons that tau release by DnaJC5 can be blocked when inhibiting Hsc70. Direct interaction of tau with DnaJC5 maybe needed as shown by mutant tau and DnaJC5 lacking interaction sites. And finally, intracellular degradation of tau by DnaJC7 counteracts tau release, as expected, by decreasing intracellular tau levels.

In the end, the authors speculate that DnaJ/Hsc70 mediated protein release is vesicle associated and could potential be a general mechanism for the release and propagation of misfolded or aggregated proteins in neurodegenerative diseases. They further speculate on synaptic co-release of tau,

synuclein, and TDP43 in complex with DnaJ/Hsc70 in response to proteasome and/or degradation impairment.

The manuscript presented contains certainly some interesting ideas and presents a new perspective of chaperone activity in neurodegeneration, but seems in a too early state of the study; unfortunately the authors did not perform the right and carefully enough experiments to be convincing about their results and the observed phenomenon being actually relevant in neurons or even in vivo. The manuscript need major restoration and, at the stage the manuscript is in, I cannot support the publication in EMBO Journal.

Specific major concerns:
Most of the data is generated using HEK cells overexpressing tau and DnaJC5; such model is a good starting point for an initial observation of potential cellular mechanisms, however, if investigating a mechanism of CNS proteins involved in neuronal function, more relevant models - even in vitro - are necessary for proper translation of the relevance of the observed phenomenon. The detailed description the authors give in HEK cells has to be verified and translated into a neuronal systems, e.g. look at tau release in dependence on DnaJC5 and Hsc70 in primary neurons. Instead only minimum translation of the DnaJC5 facilitated tau release using neuron-like cells and slice cultures were performed. The representative data showed often is not convincing (e.g. no DnaJC5 expression in Figure 3A although presumably DnaJC5+ !?).

Furthermore, the techniques used to test the hypotheses and show the mechanisms are very limited and not always appropriate, here some examples:
To show the release of tau, synuclein and TDP43 is not just a result of general protein overexpression the proper controls are missing; for example, one or two non-IDP and non-aggregating proteins should be over-expressed and monitored for release in presence of DnaJCA. Also it is claimed that DnaJC5 and Hsc70 form a complex with tau and are released as such; there is no experiment that supports this idea although it would be relatively straight forward to show the levels of DnaJC5/Hsc70 in the medium as well and further support this model by immunoprecipitation of one of the complex components. It could also easily be showed if tau and DnaJC5/Hsc70 are really released in vesicles performing a crude purification of membrane vesicles by centrifugation, and maybe doing immune-EM on the isolated vesicles; these are all standard techniques to proof the association with extracellular vesicles.

And what happens to endogenous tau from neurons or brain slices, is that released as well in vesicles in presence of DnaJC5? Overexpressing HEK cells have very different endo- and exocytosis characteristics compared to neurons, especially when they are starved in medium without serum they are known to release a lot of proteins when overexpressed.

Pre-synaptic localization of tau +/- DnaJC5 overexpression and co-localization of tau with Hsc70 is "proven" by immunohistochemistry (in only 2 sets of cultures); a simple synaptosome preparation of cultured neurons could actually show if that is true, whereas IHC is always prone for artifacts when used for quantitative intensity. The data is not convincing, especially since the synaptophysin staining is not restricted to the synapses (?) and the image insets are chosen too small to actually show what is stated.

The data is uniformly presented and analyzed as absolute released tau levels; I think it would be better to normalize these values to the actual intracellular tau expression levels since these may vary when overexpressing tau by transfection; showing the data as ratios extra:intra-cellular tau would give a clearer picture. Instead, tau is sometimes reduced or lacking intracellularly when there is (hence of course) also no tau release (e.g. Figure 3A, 5C, 6A, 7A), and it remains open and not discussed why that is the case.

Finally, if speculating about a mechanism of tau cell-to-cell transfer, experiments analyzing the transfer of tau and tau/DnaJC5/Hsc70-complexes to naïve cells should be performed.

Throughout the manuscript there is insufficient description of what has been done and what are the results; for example it is not clear which data is from HEK cells/MC17 or neurons, and it is often not very clear what kind of tau is used, what tau antibody is used for the detection, or what kind of neurons are cultured; inhibitors used for Hsc70 and the proteasome are not named and not described

in methods. There are parts in methods that should be updated because data is not shown or shown data has no methods part.

The discussion is lacking critical comparing of the results with recent published data on tau release from neurons and glia as well as tau spreading.

All models showing the role of DnaJC5/Hsc70 for tau release have no legends, and it is not clear what is what.

Minor concerns:
Figure 1B is missing significance statement.
Figure 2 needs a non-aggregating protein control.

Figure 3A has no DnaJC5 in expressing cells; significance stars are too small.

Figure 3B needs an image showing actual AAV transduction of neurons in brain slices and not glia.

Figure 3C is missing significance statement.

Figure 3D has not indicated what the white tube is or the yellow ellipticals or "alpha"; legends to explain figure details are missing throughout all models presented!

Figure 5C is not clear what shows what here; needs to be reordered so that dot blot matches Western Blot; and why is there no intracellular tau in GFP+/Hsp70 inh+ neurons?

Figure 6A shows reduced intracellular tau with Hsp70 inhibitor in absence of DnaJC5. Why? - Please discuss!

Figure 6B shows very high tau release in absence of DnaJC5 but in presence of shRNA for Hsc70. Why? - Please discuss.

Figure 7A shows reduced intracellular tau with DnaJC8 an d DnaJB1, and hence no tau release. Why? - Please discuss!
Figure 7B is missing significance statement.

Supplemental Figure 2 - not clear why this experiment has been done and is worth showing; and siginificance statement is missing.

Supplemental Figure 3 - Dot blot of released tau in presence of different DnaJC5 concentrations is al saturated; unable to even see a dose dependence if there would be one! Please replace. Also give the name of the inhibitor used and it remains unclear why that experiment has been done.

1st Revision - authors' response 14 March 2016

Referee #1

The manuscript by Fontaine and colleagues explores the relationship between DNAJC5 and extracellular protein aggregate release. The question is intriguing and relevant and as yet there is no clear understanding as to the mechanism of protein release. This is particularly relevant to many neurodegenerative disorders with spreading pathology. Unfortunately the connection between DNAJC5, Hsp70 and tau is not fully sorted out and lacks proof that the three act together.

Concerns:
Figure 1: Why is a regular western not utilized? Is the dot blot meant to convey that the extracellular tau is "aggregated"

- *The dot blot was not used to convey aggregated tau but rather because we found it the most quantitative measure besides mass spec to accurately measure the amount of extracellular tau. The dot blot avoids having to concentrate the media because we simply pull all of the media through the membrane via vacuum. That way issues with different levels of*

evaporation and volume loss during concentrating are avoided. We have however now also provided western blots of media as requested. We are in the process of developing methods to determine the structural and biophysical properties of this extracellular tau, but it seems that since even endogenous tau can exit the cell, that it is at least likely a mixture of soluble and insoluble, aggregated material.

Figure 2B : Why is there secreted synuclein in the empty vector control. In addition, It would be nice to see the where these proteins localize. All three of these proteins, tau, synuclein and TDP-43 likely localize to different cellular compartments. So how is nuclear TDP-43 getting secreted. What is the intracellular state of these proteins "soluble" aggregated.

- *Synuclein is known to be extracellular (Borghi R Neurosci Letters 2000 PMID 10841992; Sung JBC 2005 PMID:15863497) particularly when overexpressed in cultured cells, so the synuclein in the vector control likely represents basal levels. The TDP43 is over-expressed and this protein can cycle between the nucleus and cytosol. It has been shown previously that this protein can accumulate in cytosol and nuclear compartments. We have included imaging data of the overexpressing cells as requested and by ICC find most material does appear nuclear and it is not aggregated. It is possible that TDP-43 processing for this DnaJC5 mechanism requires distinct cellular components because of its primary nuclear localization (perhaps a different variant of the Hsp70 family). We might also expect that the TDP-43 that is being triaged for release is newly produced TDP-43 as it is leaving the ribosome while still in the cytosolic space. Hsc70 is known to be involved in the processing of newly translated proteins. However, since DnaJC5-mediated release of TDP-43 was not sensitive to YM-01 (Hsc70 inhibition), it suggests the former is more likely than the latter. As to the aggregated state of these proteins, since both wildtype and mutant forms are released, one would expect that the released material is either soluble or a mixture of soluble and aggregated material, since the wildtype variants of these proteins do not aggregate in standard cell models. We have addressed this in the text of the manuscript.*

Figure 3: It looks like there is more intracellular and extracellular tau. Has the level of tau experssion been confirmed as similar with and without dnajc5 in these cultures?

- *We did confirm that levels of expression were the same. The amount of extracellular tau is definitely lower than intracellular, even after DnaJC5 treatment. But DnaJC5 clearly drives it out. As you can see from the mRNA data in extended figure 2, if anything, message is increased in DnaJC5-expressing cells, confirming the intracellular reductions are due to enhanced release.*

Figure 4: it is unclear to me what this figure is telling me? it seems to me that if there was more tau at the synapses or less tau at the synapse one could make a similar conclusion.

- *We hypothesize that if there were more tau at the synapse, we would see less tau released by DnaJC5. These data show that there is less tau at the synapse in the presence of DnaJC5 overexpression. While we believe these data support the idea that tau levels are reduced at the synapse when DnaJC5 is overexpressed, we have moved this data to the Expanded view figures at the editor's suggestion and clarified our references to this data in the text.*

Karin: To me showing the co-localization at the synapse is useful and showing that you have less upon DNAJC5 OE is also of value. Could one make a similar conclusion if there was more – Is there an argument that I am missing?? Not key for the manuscript, but I would leave it in maybe as an expanded view figure.

Figure 5-7: what is the inhibitor that is used? This only shows that HSc70 is required for tau release and that DNAJC5 is required. tau needs to bind to Hsc70 and DNAJC5 needs to get to the membrane for tau release but there is no experiment or evidence that DNAJC5 interacts with Tau and HSC70 to mediate release. These data could suggest that these are two independent pathways.

- *We apologize for not giving more details about the inhibitor in the first submission. The inhibitor we used in the paper is a rhodacyanine derivative compound called YM-01 (derived from MKT-077), and these compounds are extremely well-documented in the literature as allosteric modulators of the DnaJ/Hsc70 interaction. We know that tau binds to the distinct substrate binding domain of Hsc70 through our work in 2015 (Fontaine 2015 HMG), so the tripartite DnaJc5/Hsc70/tau complex can exist. In this way, YM-01*

disrupts the DnaJC5/Hsc70 interaction (which we now provide data for), thereby facilitating tau degradation through the proteasome and possibly autophagy rather than through its release. We now include additional data supporting this as well as aligned data with an Hsc70 mutant that we know mimics the inhibitor in some ways.

Referee #2

In this manuscript Fontaine et al propose that DnaJC5, a co-chaperone, works with the molecular chaperone Hsc70 to regulate the extracellular release of tau and other neurodegenerative proteins such as TDP43 and alpha-synuclein. They report that this DnaJC5/Hsc70 mediated release is a unifying mechanism by which neurodegenerative proteins exit the cell to propagate pathology throughout the brain.

Overexpression of DnaJC5 in HEK cells leads to an increase in extracellular tau, alpha-synuclein and TDP43. Overexpression of DnaJC5 in neuronal cell lines and organotypic slice cultures leads to an increase in extracellular tau. The authors report that this DnaJC5 mediated release of tau is dependent on both tau's interaction with Hsc70 and on the activity of Hsc70 itself. Blocking both abolishes increase in extracellular tau. Finally they report that other DnaJ proteins do not affect release of tau as which occurs with DnaJC5.

Overall, the findings with HSC70 and DnaJC5 modulate the extracellular release of tau, synuclein, and TDP-43 is novel and suggest that they could be involved in the spreading of these proteins. However, only the normal, non-aggregated forms of these proteins are assessed here. These findings add importantly to this area. Several additional points need to be addressed for the data to be better interpretable.

Comments:

1. While the dot blots and western blots do show increased amount of extracellular proteins, it would be better to have a more quantitative method to better quantitate at least the results for tau. This is particularly true for analyzing intracellular tau and for making meaningful comparisons between extra and intra-cellular levels of these proteins. For example, in Fig 6B, there seems to be very little or no difference in extracellular tau (as seen in the dot blot) in the absence and presence of DnaJC5, which seems to go against the main premise of the paper. Quantifying these levels would be more accurate. In addition, showing a representative Western blot of tau, alpha-synuclein, and TDP-43 for the proteins in the extracellular space (instead of a dot blot), would be useful.

- *We have included an alternate quantification of figure 6B (now 5B), of ratios of intracellular to extracellular tau as Expanded View Figure 6.*

2. Experiments showing increases in extracellular alpha-synuclein and TDP43 were done only in HEK cells. In order for the authors to make the claim that DnaJC5/Hsc70 drive release of multiple proteins, it would be helpful to show the same experiments they did with tau for alpha-synuclein and TDP 43 in both HEK cells and a neuronal cell type.

- *We have now included data showing that α-synuclein and TDP-43 are driven out of the cell by DnaJC5 in a neuronal cell line, and that the mechanism for this release is dependent on a SNAP23-mediated exocytotic process. The mechanism for synuclein and tau appears to be similar, but TDP-43 seems to be processed slightly differently, perhaps because it is typically destined for the nucleus. We will continue to explore the subtle differences between distinct neurodegenerative disease associated clients in future studies.*

3. Comparing effects of DnaJC5 on extra and intracellular levels of tau in Fig 1 and 3B, it seems that intracellular tau levels are reduced in HEK cells but not in M17 cells. Fig 3C also seems to indicate that DnaJC5 doesn't affect intracellular tau levels in primary neurons. A similar result is seen in the organotypic slice culture experiments shown in Fig 5. Why is this? The authors should comment on this.

- *We suspect there is an overexpression effect, so that intracellular tau levels decrease when tau is exogenously overexpressed, particularly in highly transfection efficient HEK cells. This is why we went to great lengths to prove that this was not just an over-expression artifact by showing that endogenous tau is released by JC5 and also by showing that some*

over-expressed proteins are not released by JC5 over-expression. We also worried that DnaJC5 was sending some tau for degradation and some for release, which led us to look extensively at tau degradation, but DnaJC5 did not promote degradation. It seems that HEK cells are extremely sensitive to DnaJC5 mediated exocytosis. This could be because they have very low levels of this protein endogenously expressed.

4. Tau mRNA levels were only assessed in HEK cells to show that DnaJC5 mediated release of tau does not alter mRNA. Is this true for other cell types used as well? Similar analysis should be done on M17 cells as well as the other cell models used here.

- *We have now included the mRNA data for α-synuclein and TDP-43 as well in Figure EV 1.*

Referee #3

General summary and Opinion: In their presented study "DNAJ/HSc70 chaperone complexes control extracellular release of neurodegenerative associated proteins", Sarah N. Fontaine et al. report that DNAJc5 and Hsc70 synergistically mediate the release of tau in vitro. The finding that chaperones are directly involved in shutteling tau out of the cell is in intriguing finding and very interesting idea; especially in the face of recently reported release and propagation of endogenous and wild-type transgenic tau. After showing that DnaJC5 overexpression triggers the release of also overexpressed tau in HEK cells, the authors show that this is true as well for misfolding mutant tau R406W and P301L, as well as for wild-type and mutant synuclein and TDP43. They continue to show a similar effect in neuron-like M17 cells and slice cultures from wild-type mice after AAV DnaJC5 transduction and a decrease in tau release in slices from DnaJC5 knockout mice.

In primary neuronal cultures (not clear from what source) tau and Hsc70 co-localization with presynaptic markers is indicated by immunofluorescence, and further show in au overexpressing HEK cells and neurons that tau release by DnaJC5 can be blocked when inhibiting Hsc70. Direct interaction of tau with DnaJC5 maybe needed as shown by mutant tau and DnaJC5 lacking interaction sites. And finally, intracellular degradation of tau by DnaJC7 counteracts tau release, as expected, by decreasing intracellular tau levels.

In the end, the authors speculate that DnaJ/Hsc70 mediated protein release is vesicle associated and could potential be a general mechanism for the release and propagation of misfolded or aggregated proteins in neurodegenerative diseases. They further speculate on synaptic co-release of tau, synuclein, and TDP43 in complex with DnaJ/Hsc70 in response to proteasome and/or degradation impairment.

The manuscript presented contains certainly some interesting ideas and presents a new perspective of chaperone activity in neurodegeneration, but seems in a too early state of the study; unfortunately the authors did not perform the right and carefully enough experiments to be convincing about their results and the observed phenomenon being actually relevant in neurons or even in vivo. The manuscript need major restoration and, at the stage the manuscript is in, I cannot support the publication in EMBO Journal.

Specific major concerns:
Most of the data is generated using HEK cells overexpressing tau and DnaJC5; such model is a good starting point for an initial observation of potential cellular mechanisms, however, if investigating a mechanism of CNS proteins involved in neuronal function, more relevant models - even in vitro - are necessary for proper translation of the relevance of the observed phenomenon. The detailed description the authors give in HEK cells has to be verified and translated into a neuronal systems, e.g. look at tau release in dependence on DnaJC5 and Hsc70 in primary neurons. Instead only minimum translation of the DnaJC5 facilitated tau release using neuron-like cells and slice cultures were performed. The representative data showed often is not convincing (e.g. no DnaJC5 expression in Figure 3A although presumably DnaJC5+ !?).

- *We have confirmed the dependency of this mechanism on Hsc70 in non neuronal (Fig 4A,F,G), neuronal (M17- Figure B,C), and primary neurons from tau transgenic mice (Fig 3E).*
- *We have replaced the blot in Fig 3A.*

Furthermore, the techniques used to test the hypotheses and show the mechanisms are very limited and not always appropriate, here some examples:

To show the release of tau, synuclein and TDP43 is not just a result of general protein overexpression the proper controls are missing; for example, one or two non-IDP and non-aggregating proteins should be over-expressed and monitored for release in presence of DnaJCA.

Also it is claimed that DnaJC5 and Hsc70 form a complex with tau and are released as such; there is no experiment that supports this idea although it would be relatively straight forward to show the levels of DnaJC5/Hsc70 in the medium as well and further support this model by immunoprecipitation of one of the complex components. It could also easily be showed if tau and DnaJC5/Hsc70 are really released in vesicles performing a crude purification of membrane vesicles by centrifugation, and maybe doing immune-EM on the isolated vesicles; these are all standard techniques to proof the association with extracellular vesicles. And what happens to endogenous tau from neurons or brain slices, is that released as well in vesicles in presence of DnaJC5? Overexpressing HEK cells have very different endo- and exocytosis characteristics compared to neurons, especially when they are starved in medium without serum they are known to release a lot of proteins when overexpressed.

- *We now show evidence that DnaJC5/Hsc70/tau are a complex by IP, and that this complex is dependent on the interaction with DnaJC5 and Hsc70. When Hsc70 is inhibited with a small molecule, YM-01, or by a genetic modification, release is reduced (Figure 3 and 4).*
- *Currently under this given time frame we could not perform the extracellular vesicle analysis. We feel this is beyond the scope of this paper and plan to include it within our follow-up study to determine the structure and biophysical characteristics of the released material.*
- *In fact we do find that endogenous tau is released in neurons, neuronal cells, and slices by DnaJC5 (Figure 3).*

Pre-synaptic localization of tau +/- DnaJC5 overexpression and co-localization of tau with Hsc70 is "proven" by immunohistochemistry (in only 2 sets of cultures); a simple synaptosome preparation of cultured neurons could actually show if that is true, whereas IHC is always prone for artifacts when used for quantitative intensity. The data is not convincing, especially since the synaptophysin staining is not restricted to the synapses (?) and the image insets are chosen too small to actually show what is stated.

- *We thank the reviewer for this comment and while we believe these data support the idea that tau levels are reduced at the synapse when DnaJC5 is overexpressed, we have moved this data to the Expanded view figures at the editor's suggestion and clarified our references to this data in the text.*

The data is uniformly presented and analyzed as absolute released tau levels; I think it would be better to normalize these values to the actual intracellular tau expression levels since these may vary when overexpressing tau by transfection; showing the data as ratios extra:intra-cellular tau would give a clearer picture. Instead, tau is sometimes reduced or lacking intracellularly when there is (hence of course) also no tau release (e.g. Figure 3A, 5C, 6A, 7A), and it remains open and not discussed why that is the case.

- *Our data suggest that when tau is triaged for degradation, extracellular release is reduced. In this way, we suggest, in our model, that the balance of DnaJs does in fact mediate the ultimate fate of these neurodegenerative-disease associated client proteins. We have clarified the text to address this.*
- *Further, we feel that representing the absolute levels, rather than the ratio provides a clearer representation of the effects of DnaJC5 on the amount of tau, synuclein, and TDP43 in the media.*

Finally, if speculating about a mechanism of tau cell-to-cell transfer, experiments analyzing the transfer of tau and tau/DnaJC5/Hsc70-complexes to naïve cells should be performed.

- *We thank the reviewer for this comment. We feel these experiments are beyond the scope of this paper, which is focused on the pathway involved.*

Throughout the manuscript there is insufficient description of what has been done and what are the results; for example it is not clear which data is from HEK cells/MC17 or neurons, and it is often not very clear what kind of tau is used, what tau antibody is used for the detection, or what kind of neurons are cultured; inhibitors used for Hsc70 and the proteasome are not named and not described in methods. There are parts in methods that should be updated because data is not shown or shown data has no methods part.

- *We thank the reviewer for highlighting this and have updated the manuscript to better reflect the experiments and methods.*

The discussion is lacking critical comparing of the results with recent published data on tau release from neurons and glia as well as tau spreading.

- *We have expanded our discussion to include the latest studies published on tau spreading and release.*

All models showing the role of DnaJC5/Hsc70 for tau release have no legends, and it is not clear what is what.

- *We have clarified these figures and figure legends to explain the models more fully.*

Minor concerns:
Figure 1B is missing significance statement.

- *We have added the significance statement.*

Figure 2 needs a non-aggregating protein control.

- *We have a control that is not released by DnaJC5 in Fig. EV8*

Figure 3A has no DnaJC5 in expressing cells; significance stars are too small.

- *We have replaced the DnaJC5 blot with a better representation and updated the figure for readability.*

Figure 3B needs an image showing actual AAV transduction of neurons in brain slices and not glia.

- *These data are already published with our AAV method in organtypic cultures has been published previously in Fontaine 2015 JBC and Fontaine 2015 HMG.*

Figure 3C is missing significance statement.

- *We have added the significance statement.*

Figure 3D has not indicated what the white tube is or the yellow ellipticals or "alpha"; legends to explain figure details are missing throughout all models presented!

- *We thank the reviewer for pointing this oversight out and have updated the models and legends for clarity.*

Figure 5C is not clear what shows what here; needs to be reordered so that dot blot matches Western Blot; and why is there no intracellular tau in GFP+/Hsp70 inh+ neurons?

- *We have replaced the labelling to be clearer.*
- *YM-01 is a very potent inhibitor of Hsc70 that reduces tau levels by sending tau to the proteasome (Abisambra 2013 Biol Pysch), thus the reductions in intracellular tau levels.*

Figure 6A shows reduced intracellular tau with Hsp70 inhibitor in absence of DnaJC5. Why? - Please discuss!

- *YM-01 is a very potent inhibitor of Hsc70 that reduces tau levels by sending tau to the proteasome (Abisambra 2013 Biol Pysch), thus the reductions in intracellular tau levels. We have clarified the manuscript to better describe these data.*

Figure 6B shows very high tau release in absence of DnaJC5 but in presence of shRNA for Hsc70. Why? - Please discuss.

- *We have clarified these results in the manuscript.*

Figure 7A shows reduced intracellular tau with DnaJC8 and DnaJB1, and hence no tau release. Why? - Please discuss!

- *We have clarified these results in the manuscript. While both of the DnaJ proteins can reduce intracellular tau levels by tipping the balance in favor of degradation, on DnaJC5 enhances release.*

Figure 7B is missing significance statement.

- *We have rectified this oversight.*

Supplemental Figure 2 - not clear why this experiment has been done and is worth showing; and siginificance statement is missing.

- *We have clarified these results in the manuscript.*

Supplemental Figure 3 - Dot blot of released tau in presence of different DnaJC5 concentrations is al saturated; unable to even see a dose dependence if there would be one! Please replace. Also give the name of the inhibitor used and it remains unclear why that experiment has been done.

- *We have replaced the blot with a less saturated exposure, clarified in the manuscript that the reason for this experiment is to prove that DnaJC5 is not enhancing degradation but release specifically and have clarified the figure by including the name of the inhibitor in the figure.*

2nd Editorial Decision 04 April 2016

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by the original referees #1 and 2 and their comments are provided below. As you can see, both referees appreciate the introduced changes and support publication here. There are just a few minor revisions needed - nothing major. Once I get the revised version back I will accept the manuscript for publication here.

REFEREE REPORTS

Referee #1:

This is a revised manuscript from Fontaine and colleagues. Teh manuscript evaluates a novel molecular complex that facilitates the release of intracellular proteins associated with neurodegenerative disease processes. The revised manuscript has addressed many of my initial concerns and most notably both genetically and chemically demonstrated that a complex of Tau-HSC70-DNAJC5 facilite extracellular release of neurodegenerative proteins.

Comments:Figure 2B. It is still unclear to me how a-syn can be released from HEK293 cells when it is not overexpressed and even detected as intracellular in the first lane. Are the authors suggesting that undectable endogenous a-syn is now detectable in the extracellular fraction without it being overexpressed as in lanes 2-3 and lanes 5-6?

Use YM-01 instead of HSP70 inhibitor throughout the figures for consistency.

Referee #2:

I think the authors have done a nice job answering this reviewer's previous critique. The only issue is that in answering whether DNAJC5 affects tau, TDP-43, or synuclein mRNA levels, they answered that it does not and refer to figure EV2. However, in Figure EV2, it shows that DNAJC5 increases tau and synuclein mRNA. There must be a mistake with the figure? This needs to be addressed.

2nd Revision - authors' response 25 April 2016

Referee #1:

Figure 2B. It is still unclear to me how a-syn can be released from HEK293 cells when it is not overexpressed and even detected as intracellular in the first lane. Are the authors suggesting that undectable endogenous a-syn is now detectable in the extracellular fraction without it being overexpressed as in lanes 2-3 and lanes 5.6?

- *There are reasonable levels of endogenous synuclein present in HEK cells, which also are released upon DnaJC5 over-expression, they are just not nearly as high as those in comparison to when synuclein is over-expressed. This is what we were trying to convey with this figure as shown, by saturating the exposure. Now we provide two exposures, one low and one high to address this important point. When the exposure is lowered, it is clear that endogenous synuclein release is less abundant than over-expressed synuclein.*

Use YM-01 instead of HSP70 inhibitor throughout the figures for consistency.

- *We can certainly make this change, but thought that a non-expert reader might find the term Hsp70 Inhibitor easier to understand than YM-01. Karin, it is up to you.*

Referee #2:

I think the authors have done a nice job answering this reviewer's previous critique. The only issue is that in answering whether DNAJC5 affects tau, TDP-43, or synuclein mRNA levels, they answered that it does not and refer to figure EV2. However, in Figure EV2, it shows that DNAJC5 increases tau and synuclein mRNA. There must be a mistake with the figure? This needs to be addressed.

- *We apologize for this mistake. We indeed made a mistake with these figures. The corrected graph is now provided.*

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sarah N. Fontaine and Chad A. Dickey
Journal Submitted to: EMBO Journal
Manuscript Number:

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least three litters were harvested for primary neurons or organotypic cultures were performed to ensure a sufficient n of mice of the correct genotype would be analyzed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Primary neurons were prepared from 3-5 litters of pups for transgenic mouse lines, with a minimm of 3 pups per genotype per litter typically. Neurons were obtained from three litters of pups for experimentation with wild type mice. Similar litter numbers were used for organtypic slice cultures. Typically 8 slices from 3 pups of each genotype were analyzed for organotypic experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Inclusion criteria included animals of both sexes for primary neuron and organotypic preparations. For transgenic animals, analyses were performed per genotype. Any data more than two standard deviations from the mean were excluded; these criteria were established prior to experimentation.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Tissues for primary neuron and organotypic cultures was isolated in a randomized fashion as genotypes were determined after tissue harvest.
For animal studies, include a statement about randomization even if no randomization was used.	Tissues for primary neuron and organotypic cultures was isolated in a randomized fashion as genotypes were determined after tissue harvest.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For imaging analysis, all quantifications analyses were peformed by an independent researcher who did not take the image to minimize bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Tissues for primary neuron and organotypic cultures was isolated in a randomized fashion as genotypes were determined after tissue harvest.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. To ensure that any differences were in fact due to differences in the groups and not just random variance, ANOVA tests were performed with Tukey's posthoc analyses to ensure comparisons between groups.
Is there an estimate of variation within each group of data?	All stastical tests were performed using a normal distrubition model.
Is the variance similar between the groups that are being statistically compared?	The variance between groups was similar.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibody
http://1degreebio.org	1Degree
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-reporting-the-arrive-gui	ARRIVE G
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guid
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Gui
http://ClinicalTrials.gov	Clinical T
http://www.consort-statement.org	CONSOR
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSOR
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour-marker-prognc	REMARK
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomode
http://biomodels.net/miriam/	MIRIAM
http://jjj.biochem.sun.ac.za	JWS Onli
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecur
http://www.selectagents.gov/	List of Se

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Monoclonal flag (mouse) Sigma-Aldrich F1804 CSPa Synaptic Systems 153003 Synaptophysin, Synaptic Systems, 101002 SNAP23, Synaptic Systems, 111202 SNAP25, Synatpic Systems, 111002 Tau 12 L. Binder, also MAB2241 from Millipore Tau 5 L. Binder, also ab80579 from Abcam H150 tau, Santa Cruz Biotechnology, sc-5587 Tau V20, Santa Cruz Biotechnology, sc-1996 TDP43, Cell Signaling Technology, 3448S A synuclein Cell Signaling Technology, 2542S Hsc70, Enzo ADI-SPA-815-D PHF1, Peter Davies, Am J Clin path 1986 85:381 Dickson DW Gapdh ,Cell Signaling Technology 2188S Actin, Cell Signaling Technology, 8457S
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were obtained from ATCC

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mice of the following strains: C57Bl/6 (Jackson Laboraties), Cspa+/- (T. Sudhof, Stanford University) and P301Mapt(Jackson Laboratories) were housed in static cages with enrichement in a 12h light/dark cycle with ad libidium food and water. All animal proceedures and housing were approved by USF IACUC. Primary neruons were obtained from E16 embryos. Organotypic cultures were prepared from mice of both sexes at p14-21. This was done as CSPa-/- mice could be identified phenotypically at this age to ensure sufficient sample size for experimentation.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal studies were performed in accordance to the guidelines and subjected to the oversight of the University of South Florida Institutional Animal Care and Use Committee.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subjects were used in this study.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (See link list at top right) or EGA (See link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A